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Stationary biofilm growth normalizes mutation frequencies and mutant prevention concentrations in *Pseudomonas aeruginosa* from cystic fibrosis patients

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Abstract

Bacterial biofilms play an important role in the persistent colonization of the respiratory tract in cystic fibrosis (CF) patients. The trade-offs among planktonic or sessile modes of growth, mutation frequency, antibiotic susceptibility and mutant prevention concentrations (MPCs) were studied in a well-defined collection of 42 CF *Pseudomonas aeruginosa* isolates. MICs of ciprofloxacin, tobramycin, imipenem and ceftazidime increased in the biofilm mode of growth, but not the MPCs of the same drugs. The mutation frequency median was significantly higher in planktonic conditions (1.1×10^{-8}) than in biofilm (9.9×10^{-9}) (p 0.015). Isolates categorized as hypomutable increased their mutation frequency from 3.6×10^{-9} in the planktonic mode to 6×10^{-8} in biofilm, whereas normomutators (from 9.4×10^{-8} to 5.3×10^{-8}) and hypermutators (from 1.6×10^{-6} to 7.7×10^{-7}) decreased their mutation frequencies in biofilm. High and low mutation frequencies in planktonic growth converge into the normomutable category in the biofilm mode of growth of CF *P. aeruginosa*, leading to stabilization of MPCs. This result suggests that once the biofilm mode of growth has been established, the propensity of CF *P. aeruginosa* populations to evolve towards resistance is not necessarily increased.

Keywords: Biofilm, cystic fibrosis, mutant prevention concentrations, mutation frequency, *Pseudomonas aeruginosa*
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Introduction

Biofilms are complex and organized bacterial communities that are able to grow in association with different biological or inert surfaces. The clinical consequences of the biofilm mode of growth of pathogenic bacteria relate to the difficulty of therapeutic eradication of sessile cells forming such supracellular structures [1,2]. Bacterial biofilms play an important role in the persistent colonization of the respiratory tract in cystic fibrosis (CF) patients, and have been associated with a significant increase in antibiotic resistance [3–6]. Another important feature of CF pathogens is hypermutability, a phenomenon that confers a selective advantage, as the bacteria are often exposed to a high level of immune response and high doses of antibiotics for prolonged periods [7,8]. Hypermutable *Pseudomonas aeruginosa* isolates are frequently recovered from the lungs of CF patients [7]. More recently, it has been shown that the biofilm mode of growth might increase the mutation frequency of the reference strain *P. aeruginosa* PAO1, although this possibility has not yet been explored with clinical isolates [9].

On the other hand, the mutant prevention concentration (MPC) is the lowest antibiotic concentration that inhibits the growth of single resistant mutants emerging within a high-density bacterial population. The MPC estimates the ability of each antibiotic to select resistant mutants, and cannot be predicted from the MIC [10,11]. By comparison of MPCs and MICs, a mutant selection window can be defined, showing a range of concentrations at which resistant mutants emerged. This concept has clinical applications in CF [12,13]. Theoretically, the selection window is expected to be wider in hypermutable isolates, as the diversity of mutants (particularly for double mutants) is larger. The aim of this work was

to explore the possible differences in mutation frequency of cells with planktonic and biofilm modes of growth in a collection of CF *P. aeruginosa* isolates and their influence on MPCs.

Materials and Methods

Bacterial isolates

Forty-two *P. aeruginosa* clinical isolates from sputum samples of ten patients from our CF Unit (2002–2006) were studied (two to nine isolates per patient). Selection of isolates was carried out in order to ensure a collection representing CF isolates with the following criteria: (i) different pulsotype; (ii) different colonial morphotype; (iii) different year of isolation, if belonging to a particular patient; and (iv) as far as possible, representing each one of the three mutation frequency categories in the planktonic mode of growth. Genetic diversity was determined by pulsed-field gel electrophoresis (PFGE)–*Xba*I and UPGMA dendrogram construction (Phoretrix 5.0 software; Bio-Rad, Hercules, CA, USA). Relationship among the isolates was considered when the Dice coefficient was ≥0.7.

Mutation frequency determination

Independent triplicate 10-mL overnight Mueller–Hinton broth (Difco, Detroit, MI, USA) cultures of each *P. aeruginosa* isolate were centrifuged and resuspended in 1 mL of of saline solution to ensure a number of cells exceeding 10^9 CFU/mL. Serial ten-fold dilutions were plated in LB agar (Difco) to determine the number of viable cells, and 0.5 mL was seeded into LB agar plates with 300 mg/L rifampicin. Plates were incubated (48 h), and the total number of mutant colonies was determined. The mutation frequency was defined as the median number of colonies of mutants divided by the median number of total viable cells obtained in the different tubes. If discordance between median and mean values was high, cultures were replicated in triplicate [14]. Isolates were classified as hypomutators (*f*-range: 5×10^{-10} to 5×10^{-9}), normomutators (7.5×10^{-9} to 7.5×10^{-8}), and hypermutators (1×10^{-7} to 5×10^{-6}), as previously defined [15]. Mutation frequencies for biofilm formation were determined similarly, but using bacterial cells from nitrocellulose mature biofilms as the incoculum [16]. This method was used to ensure an equivalent inoculum to the planktonic conditions (at least 10^9 CFU/mL). In short, biofilm was developed for 3 days at 37°C on a nitrocellulose filter disk inoculated with 100 μL of an overnight LB broth culture of known planktonic mutation frequency, and placed on an LB agar plate. The bacteria growing on the filter surface (biofilm bacteria) were suspended in saline solution, homogenized with vortexing, and plated in the LB agar plates containing rifampicin (300 mg/L). The efficiency of vortex homogenization was ascertained by light microscopy (less than one bacterial clump per ×1000 optical field). The density of viable cells was calculated with the use of ten-fold dilutions; only biofilms with an initial concentration of 10^9–10^{10} CFU/mL were considered. The *P. aeruginosa* PAO1 normomutator strain and the hypermutator PAO1Δ*mutS* derivative were used as controls [17].

Antibiotic susceptibility testing

Susceptibility to ciprofloxacin, tobramycin, imipenem and ceftazidime was determined by standard microdilution [18]. *P. aeruginosa* ATCC 27853 was used as the control strain in each run. Breakpoint susceptibility criteria are given in Table 1 [19]. The biofilm MIC susceptibility assay was performed using a flat-bottomed 96-well microtitre plate (catalog no. 269787; Nalgene Nunc International, Rochester, NY, USA). Bacterial biofilms were formed by immersing the pegs of a modified polystyrene microtitre lid (catalog no. 445497; Nunc TSP system) into this biofilm growth plate. Peg lids were rinsed three times in sterile water, placed onto flat-bottomed microtitre plates containing two-fold dilutions of antibiotic, and incubated for 18–20 h at 37°C [20].

MPC

For MPCs in the planktonic mode of growth, 0.5 mL of an overnight shaking culture in 10 mL of LB broth containing 10^9–10^{10} CFU/mL were seeded in LB agar plates supplemented with serial dilutions of antibiotics. Plates were incubated at 37°C for 48 h, and the MPC corresponded to the lowest concentration with non-visible growth. The Moskowitz method [20] was unsuitable for determining the MPC in mature biofilm, as this technique does not guarantee the heavy inoculum required. Consequently, biofilms were formed in nitrocellulose filters deposited over LB agar plates [16]. After 3 days of incubation at 37°C, all of the bacteria on the filter were suspended in 3 mL of saline. Under these conditions, the cell density was comparable to that of planktonic growth (10^9–10^{10} CFU/mL), and 0.5 mL of the suspension was seeded in antibiotic-supplemented plates as described above. Both media and incubation conditions were identical for planktonic growth and biofilm, but the inoculum was different: for planktonic growth, a 24-h 10-mL LB broth inoculum was used, whereas for biofilm, 3 days of mature growth in a nitrocellulose disk was used. This difference was attributable to the high cell density required for the MPC determination.

TABLE 1. MIC and mutant prevention concentration (MPC) distribution for the 42 *Pseudomonas aeruginosa* isolates, and overall susceptibility rates in both planktonic and biofilm conditions

	Antibiotic dilution (mg/L)																
	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	$Value_{50}$	$Value_{90}$	% S	% R
Ciprofloxacin																≤1 mg/L[a]	≥4 mg/L[b]
MIC	1	2	11	9	8	9	2							2	8	33.3	45.2
MIC in biofilm		1	3	9	8	10	11	12	1					4	16	11.1	69.0
MPC				2	6	9	12	12	1					16	32		
MPC in biofilm					15	10	9	7	1					8	32		
Tobramycin																≤4 mg/L[a]	≥16 mg/L[b]
MIC			6	6	10	14	2	4						4	16	52.3	
MIC in biofilm		2	1	4	10	8	7	2	4	2	2			8	64	40.5	14.3
MPC						4	13	8	13	4				32	64		40.5
MPC in biofilm						5	5	12	8	11	1			32	128		
Imipenem																≤4 mg/L[a]	≥16 mg/L[b]
MIC			1	10	5	11	7	5	3					8	32	38.1	35.7
MIC in biofilm					4	5	5	10	11	5	2			32	128	9.5	78.6
MPC							2	9	21	9	1			64	128		
MPC in biofilm								13	15	6	5	3		64	256		
Ceftazidime																≤8 mg/L[a]	≥32 mg/L[b]
MIC					4	10	10	8	2	4	1	3		16	128	33.3	42.8
MIC in biofilm				2	2	2	5	2	1	1	7	9	11	128	1024	14.3	73.8
MPC								1	2	4	4	9	22	1024	1024		
MPC in biofilm								3	3	8	5	17	6	512	1024		

R, resistant; S, susceptible. a and b: susceptability and resistant criteria, respectively [19].

Statistical analysis

All data were obtained from triplicates of separate experiments. Continuous data were expressed as means and standard deviations; categorical data were expressed as relative and absolute frequencies, and median values were used for them. To investigate whether the differences in mutation frequencies between biofilm and planktonic growth were associated with baseline frequency of mutation, a linear regression analysis was used, with the logarithm of the difference in mutation rate between biofilm and planktonic growth as a dependent variable and the (baseline) frequency of mutation as an independent variable. Paired comparisons of MIC and MPC distributions between the different types of growth (biofilm and planktonic) were performed (Wilcoxon signed rank sum test). Classes of mutation frequency (hypomutators, normomutators or hypermutators) were also compared (Kruskall–Wallis test). The significance level was 0.05. For multiple *post hoc* pairwise comparisons, a Bonferroni corrected significance level was used (α = 0.016).

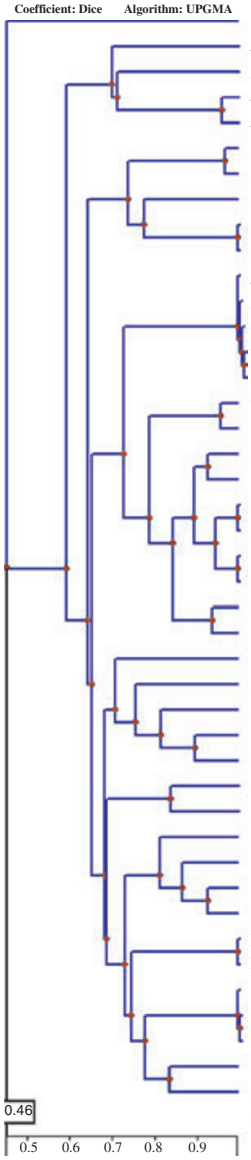
Results

Genetic and phenotypic diversity of *P. aeruginosa* isolates

Forty of the 42 CF *P. aeruginosa* isolates were clustered in 18 PFGE patterns or pulsotypes (A–R), and two isolates (21 and 36) were consistently untypeable (Fig. 1). Morphotypes were determined on Columbia agar with 5% sheep blood, and were as follows: mucoid (23 isolates, 55%), enterobacterial (seven isolates, 17%), dwarf (five isolates, 12%), metallic (four isolates, 9%) and rough (three isolates, 7%).

Mutation frequency in planktonic and biofilm conditions

Rifampicin mutation frequencies (*f*) in planktonic conditions allowed us to classify our 42 isolates as hypomutators (13 isolates, 31%) normomutators (21 isolates, 50%) and hypermutators (eight isolates, 19%). Similar distributions of PFGE patterns and morphotypes were observed in all three categories (Fig. 1). When biofilm conditions were applied, 18 isolates were hypomutators (43%), 13 were normomutators (31%) and 11 were hypermutators (26%) (Figs 1 and 2). A detailed distribution of frequencies of mutation per isolate, in planktonic or biofilm conditions, is shown in Fig. 3. Linear regression analysis, based on the difference in logarithm mutation frequency values between planktonic and biofilm growth for each strain, showed that the overall mutation frequency of all 42 isolates growing in biofilm was significantly lower than in planktonic mode (p 0.015). The Wilcoxon test also detected significant differences depending on the initial (planktonic) class of mutation frequency (hypomutators, normomutators or hypermutators). As shown in Fig. 3d, hypomutatable isolates had negative results in the planktonic minus biofilm values, whereas for normomutable and hypermutable isolates, this difference was always positive. Therefore, isolates that were hypomutable in planktonic conditions significantly increased their mutation frequency values in biofilm. In contrast, normomutable and hypermutable isolates tended to reduce their mutation frequency in biofilm (Figs 2 and 3). Mutation frequencies in planktonic and biofilm conditions



Isolate	Pulsotype	Patient	Year	Morphotype	Mutation Frequency Category: Planktonic	Mutation Frequency Category: Biofilm
14	L	P2	2002	Enterobacteriaceae	Nomomutator	Nomomutator
40	M	P1	2002	Enterobacteriaceae	Hypermutator	Hypermutator
ATCC						
41	E	P10	2002	Rough	Nomomutator	Nomomutator
42	E	P10	2002	Mucoid	Normomutator	Hypomutator
34	F	P8	2004	Mucoid	Hypermutator	Hypermutator
35	F	P8	2005	Mucoid	Hypermutator	Hypermutator
32	N	P7	2003	Rough	Hypermutator	Hypermutator
27	G	P4	2006	Mucoid	Normomutator	Normomutator
28	G	P4	2006	Mucoid	Hypermutator	Hypermutator
4	B	P2	2003	Enterobacteriaceae	Hypomutator	Hypomutator
5	B	P2	2003	Enterobacteriaceae	Normomutator	Normomutator
6	B	P2	2003	Mucoid	Normomutator	Hypomutator
7	B	P2	2003	Mucoid	Hypomutator	Normomutator
8	B	P2	2003	Enterobacteriaceae	Normomutator	Normomutator
9	H	P2	2003	Mucoid	Normomutator	Normomutator
11	H	P2	2002	Mucoid	Hypomutator	Hypomutator
3	A	P2	2003	Mucoid	Normomutator	Hypomutator
10	A	P1	2003	Metalic	Normomutator	Normomutator
29	A	P1	2006	Draft	Hypomutator	Hypomutator
30	A	P1	2006	Mucoid	Normomutator	Hypomutator
1	A	P1	2004	Metalic	Hypomutator	Normomutator
22	A	P1	2006	Metalic	Hypomutator	Hypomutator
2	A	P1	2004	Mucoid	Hypomutator	Normomutator
13	A	P1	2002	Draft	Hypomutator	Hypomutator
12	O	P1	2002	Mucoid	Normomutator	Normomutator
36	P	P9	2004	Rough	Hypermutator	Hypermutator
33	Q	P7	2003	Mucoid	Hypomutator	Hypermutator
15	I	P3	2003	Enterobacteriaceae	Normomutator	Hypomutator
26	I	P3	2003	Mucoid	Normomutator	Hypomutator
37	J	P9	2004	Mucoid	Normomutator	Hypomutator
39	J	P10	2002	Draft	Hypermutator	Hypermutator
31	R	P5	2006	Mucoid	Normomutator	Hypermutator
19	C	P5	2003	Mucoid	Hypomutator	Hypomutator
17	C	P5	2003	Draft	Normomutator	Hypomutator
20	C	P5	2003	Mucoid	Normomutator	Hypomutator
Mut					Hypermutator	Hypermutator
PAO					Normomutator	Normomutator
16	D	P4	2003	Enterobacteriaceae	Normomutator	Hypomutator
18	D	P4	2003	Mucoid	Normomutator	Hypomutator
23	D	P6	2003	Mucoid	Hypomutator	Normomutator
24	K	P6	2006	Draft	Hypomutator	Normomutator
25	K	P6	2006	Mucoid	Hypomutator	Hypomutator
21	NT	P1	2006	Mucoid	Normomutator	Hypermutator
38	NT	P4	2002	Mucoid	Hypermutator	Hypermutator

FIG. 1. UPGMA dendrogram with the generic relationship among the 42 *Pseudomonas aeruginosa* cystic fibrosis isolates and the control strains. NT, non-typeable.

were 2.5×10^{-8} and 7.9×10^{-9} for the PAO1strain, respectively, and 1×10^{-6} and 8×10^{-7} for the PAO1Δ*mut*Sstrain, respectively (Fig. 3). Data were also analysed considering the morphotypes and pulsotypes but differences were not found.

MICs in planktonic and biofilm conditions

Consistently lower antibiotic resistance rates were detected among the isolates in planktonic conditions than in biofilm conditions: ciprofloxacin, 45% vs. 69%, p 0.001; tobramycin, 14% vs. 40%, p 0.01; imipenem, 36% vs. 79%, p 0.001; and ceftazidime, 43% vs. 74%, p 0.001) (Table 1; Fig. 4). Overall median MICs increased in biofilm conditions for ciprofloxacin (×3), imipenem (×4), tobramycin (×1.3) and ceftazidime (×16). Isolates showing a hypermutator phenotype in planktonic conditions had median MICs that were not significantly higher than those of isolates categorized as normomutable and hypomutable in the same planktonic conditions. The increase in MICs from planktonic to biofilm conditions was less pronounced in hypomutable and normomutable isolates (Fig. 4). In the PAO1 control strain, the biofilm mode of growth increased ceftazidime and imipenem MICs (>4); the increase was less pronounced for ciprofloxacin and

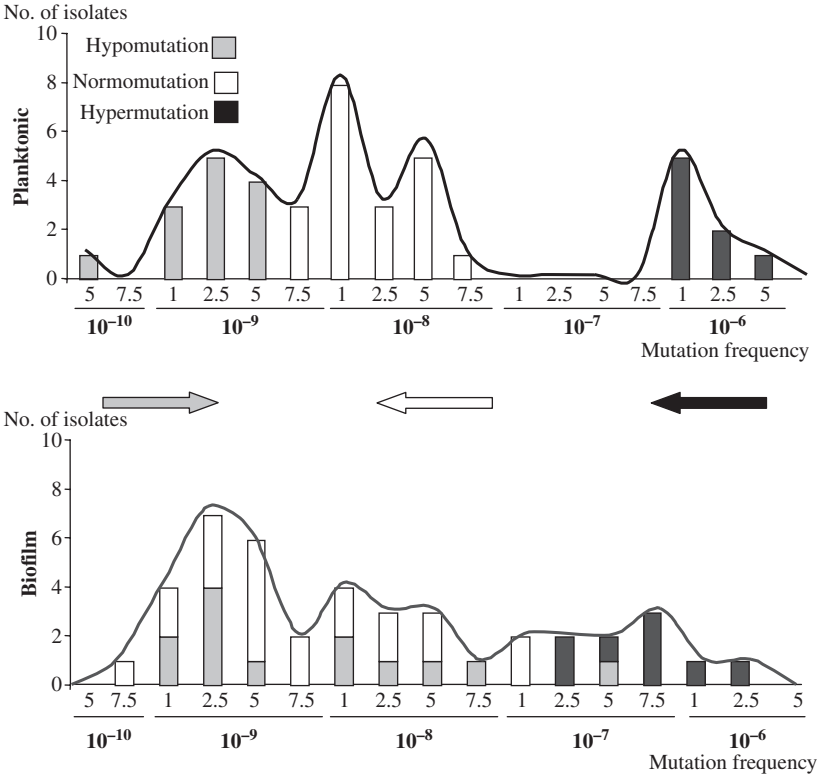


FIG. 2. Distribution of the mutation frequencies of all isolates in both planktonic and biofilm conditions. Arrows indicate major changes in mutation frequencies from the planktonic to the biofilm mode of growth. The convergence of hypermutable and hypomutable isolates towards normal mutation frequencies is presented. Bars indicate the number of hypomutators (grey), normomutators (white) and hypermutators (black) classified as having a planktonic mode of growth.

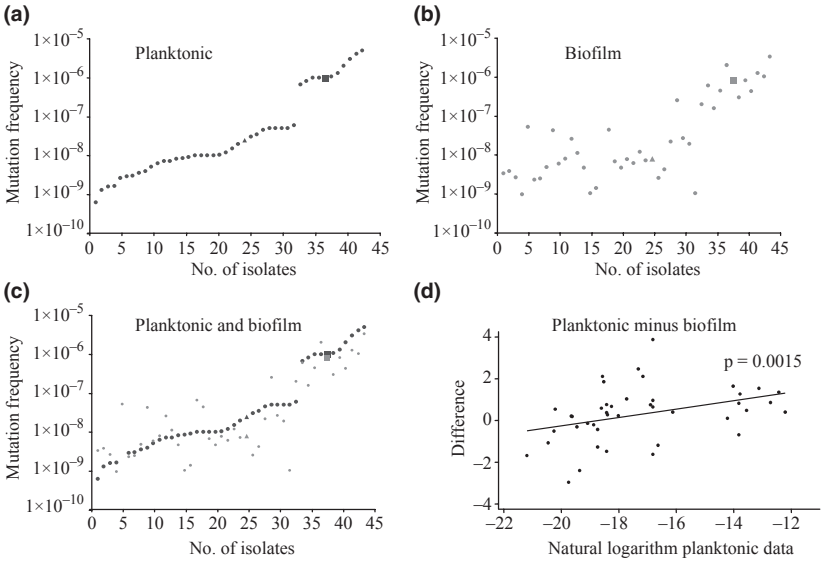


FIG. 3. Mutation frequencies for the 42 clinical isolates and the two control strains (PAO1, represented as a triangle, and PAO1Δ*mutS*, represented as a square) in planktonic conditions (black) (a), biofilm (grey) (b), and both modes of growth (c), and the difference between planktonic values and biofilm values (d).

tobramycin (≤4) (Table 2). Almost identical values were obtained for PAO1Δ*mutS*.

MPCs in planktonic and biofilm conditions

Considering all isolates, MPC_{90}s in planktonic/biofilm conditions were: ciprofloxacin, 32/32 mg/L; tobramycin, 64/128 mg/L; imipenem, 128/256 mg/L; and ceftazidime 1024/1024 mg/L. This suggests that biofilm formation does not influence, or eventually only marginally increases, MPCs (Table 1). Statistical differences were not found, although a trend towards lower MPCs in biofilm was observed for ciprofloxacin and ceftazidime, whereas for tobramycin and

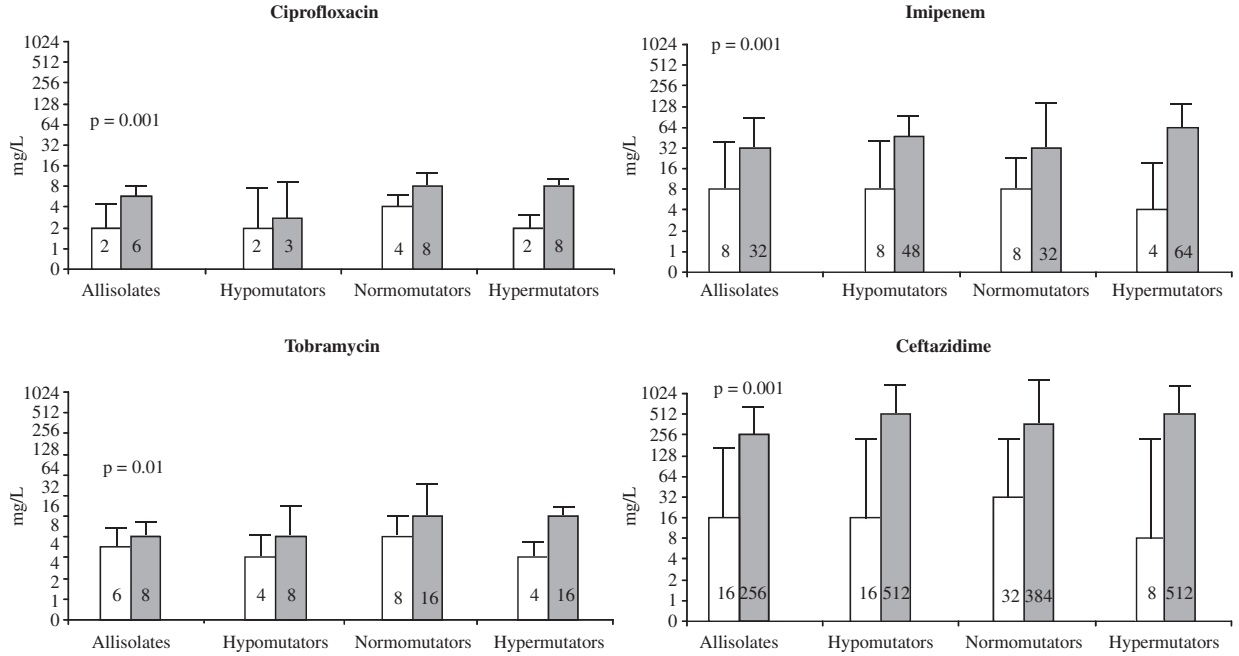


FIG. 4. Median MIC values in the planktonic (white) and biofilm (grey) conditions.

TABLE 2. MIC and mutant prevention concentration (MPC) values for the control *Pseudomonas aeruginosa* isolates

Antibiotic	Control strain	MIC (mg/L)		MPC (mg/L)	
		Planktonic	Biofilm	Planktonic	Biofilm
Ciprofloxacin	PAO1	0.25	0.25	8	4
	PAO1Δ*mutS*	0.25	0.5	16	4
Tobramycin	PAO1	1	4	128	64
	PAO1Δ*mutS*	4	16	128	64
Imipenem	PAO1	4	128	32	64
	PAO1Δ*mutS*	4	128	32	64
Ceftazidime	PAO1	2	≥1024	32	≥1024
	PAO1Δ*mutS*	8	≥1024	128	≥1024

imipenem, MPCs were almost identical to those of planktonic conditions (data not show). For the control PAO1 and PAO1Δ*mutS* strains, these values were 8/4 mg/L for ciprofloxacin, 128/64 mg/L for tobramycin, 32/64 mg/L for imipenem and 32/≥1024 mg/L for ceftazidime, again suggesting a low effect of biofilm growth on MPCs, except in the case of ceftazidime (Table 2). MPCs were not statistically different for the hypomutable, normomutable and hypermutable isolates.

Discussion

A biofilm mode of growth, hypermutability and the coexistence of different colonial morphotypes characterize *P. aeruginosa* isolates from CF patients [7,21,22]. Much less is known about the consequences of the interactions among these biological traits, particularly concerning on antibiotic susceptibility. In this work, we explored the potential differences in mutation frequencies, MICs and MPCs in a collection of *P. aeruginosa* isolates from CF patients under planktonic and sessile growth conditions.

According to conventional knowledge, bacteria in biofilms (predominantly in stationary phase) should be in a state of transient genetic instability in relation to the RpoS-dependent stress response, thus increasing mutation frequencies [23]. Previous observations in our laboratory indicate that hypermutation might increase the spontaneous availability of mutants of *P. aeruginosa*, so that if MICs are determined after the bacteria have been in antibiotic-containing broth for more than 24 h, mutants might be selected, giving a false image of resistance in an otherwise predominantly susceptible population [24]. Hypermutators might produce a further increase in the number of mutations able to potentially provide antibiotic resistance, and consequently the concentration preventing the selection of resistant mutants (the MPC). Our results suggest that this is not necessarily the case, particularly when different growth conditions are considered.

Considering all studied isolates, the mutation frequencies with regard to rifampicin in planktonic conditions were significantly higher than those obtained in biofilm. However, isolates classified as hypomutators under planktonic conditions showed a significant increase in their mutation frequency in the sessile mode of growth ($p \leq 0.015$), and most

behaved as normomutators. In contrast, isolates grouped as hypermutators in planktonic conditions tended to decrease their mutation frequency and also converted to the normomutator category. To our knowledge, this is the first time that a consistent decrease in mutation frequencies associated with biofilm formation has been observed. Our results also indicate that, at least under our experimental conditions, the stationary mode of growth of CF *P. aeruginosa* isolates, resembling that of biofilms, does not result in a higher number of mutants. This effect was less obvious, but might be present, in laboratory control strains. Driffield *et al.* documented an increase in the rifampicin and ciprofloxacin mutation frequency of the PAO1 strain in biofilms [8]. These authors used a biofilm formed on a modified Sorbarod apparatus, whereas we used a nitrocellulose filter to obtain adequate stationary inocula for the experimental conditions [16]. In our study, with a more homogeneous cell suspension and with a different rifampicin concentration (300 mg/L vs. $4 \times$ MIC) [8], we were unable to observe significant increases in mutation frequencies for the PAO1 strain.

Numerous studies have focused on antimicrobial susceptibility differences between the planktonic and biofilm modes of growth, demonstrating that isolates are more resistant in biofilms, with the exception of macrolides [25–27]. Our study also showed higher resistance rates in biofilms than in planktonic conditions, but the overall increase in MICs was not substantially higher for hypermutable isolates, and this cannot be explained by higher basal MICs. The results obtained in this study concerning the shift in mutation frequencies related to mode of growth might provide the reason for this observation, because, in biofilm, there is a convergence of both hypermutable and hypomutable isolates towards normomutable phenotypes (Fig. 4). These results were initially misleading, as the observed increase in MICs in biofilms was not higher, as would be expected, for the hypermutable organisms [7]. This was because, a proportion of hypermutable isolates tend to present a normomutable phenotype in biofilm. Planktonic hypermutable isolates do not have increased MICs in biofilm. Only isolates that maintain the hypermutable phenotype in biofilm have increased MICs.

The main caveats regarding our study are based on the use of mutation frequencies (instead of mutation rates), potential biases related to the biofilm model, and different incubation times for planktonic and biofilm inocula. Moreover, differences in growth phase might influence the mutation frequencies. We used 3 days of culture in stationary phase, which probably results in a different population structure than overnight cultures. However, our collection ensures that the different mutation frequency phenotypes were represented. The reason why there is a possible decrease in mutation frequencies in biofilm is debatable. Our results suggest that there might be an excessive cost of harbouring particular resistance mutations in biofilm, and this cost tends to be higher in hypermutable isolates. The classic observation of greater antibiotic susceptibility in *P. aeruginosa* mucoid colonies (which are more prone to form biofilms) partially supports this statement [28]. Biofilm in CF does not necessarily increase the development of inheritable antibiotic resistance, and hypermutation and high-level resistance evolve more frequently among planktonic bacteria, as a result of their susceptibility to antimicrobial agents. Accordingly, MPCs were stable in biofilm. This observation suggests that once MPCs have been exceeded, overdosage of antibiotics during therapy of CF patients is not necessarily required.

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Transparency Declaration

All authors declare no conflicts of interest.

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